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Research article

Laboratory evaluation of novel long-lasting insecticidal nets on *Aedes aegypti* L., using a high-throughput screening system

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Abstract

Vector-borne diseases, causing more than one million human deaths annually, account for more than 17% of all infectious diseases, especially malaria and dengue. Long-lasting insecticidal nets (LLINs) remain the mainstay of malaria vector control; however, the impact of LLINs on vector populations is unclear. In this study, the efficacy of LLINs was assessed under laboratory conditions against *Aedes aegypti*, using a high-throughput screening system (HITSS). Two different types of LLINs—LN A (candidate net) and LN B (reference net)—with unwashed and washed conditions were evaluated for three assays—contact irritancy assay, spatial repellency assay and toxicity response assay. The results showed that *Ae. aegypti* populations elicited a significant ($p = 0.0022$) escape response (contact irritancy) across all LLIN tests. Weak spatial repellency for all tests was observed. *Aedes aegypti* exposed to LN B (reference net) displayed a higher number of mosquito-escapees compared to LN A (candidate net). Washed LN B had a higher number escape (4.16±1.09 – 5.83±1.32) than unwashed LN B (4.17±0.79 – 5.33±1.38). Toxicity response was documented for all tested LLINs. The highest mortality was showed in washed LN B (100%). The results showed that two actions of LLINs were contact irritancy and toxicity.

Introduction

Dengue is one of the serious mosquito-borne diseases that remain public health issues in urban and suburban areas in the Americas, Asia, the Eastern Mediterranean, Africa and the Western Pacific (Messina et al., 2014). Dengue virus belongs to the Genus *Flavivirus* which comprises at least four serotypes namely Den-1, Den-2, Den-3, and Den-4 (Kumaria, 2010). It is transmitted by the bite of infective female *Aedes aegypti* L. and *Aedes albopictus* (Skuse) (Ferreira-de-Lima & Lima-Camara, 2018). Primary infection usually results in milder illness, while more severe symptoms occur in cases of repeated infection with different serotypes (Supradish et al., 2011). Almost 4 billion people in 128 countries are at risk of dengue infection spreads throughout more than one-half of the world's population (Brady et al., 2012; World Health Organization, 2017).

As no effective or commercial multi-valent dengue vaccine is readily available, prevention of this disease remains almost entirely dependent on various methods of vector control with control measures of mosquito-vectors remaining the most effective means of reducing virus transmission potential (Gubler, 1998; World Health Organization, 2012). Unfortunately, *Ae. aegypti* has proven very difficult to control because of its close association with humans and its exploitation of domestic and peridomestic environments. The standard control techniques are based on mechanical, chemical, and biological methods, including larval habitat control or elimination, and using more expensive approaches with chemical or biological means (Beier et al., 2008).

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Many chemical compounds, including organophosphates, carbamates, synthetic pyrethroids and so-called bio-rational pesticides such as bacterial toxins and insect growth regulators have long been used in national public health vector control programs. Several synthetic pyrethroids (for examples, deltamethrin, permethrin and cyfluthrin) have been introduced into the public market in recent years for controlling household nuisance mosquitoes including *Ae. aegypti* (Chareonviriyaphap et al., 1999; Somboon et al., 2003). The World Health Organization Pesticide Evaluation Scheme (WHOPES) reviews and makes recommendations on new pesticide technologies for public health programs with the most widely accepted being a product from long lasting insecticidal nets (LLINs). LLINs employ novel techniques or incorporate insecticide directly into the fabric of the net. Three insecticides of the pyrethroid group are recommended by the World Health Organization (WHO) for LLINs—permethrin, alpha-cypermethrin and deltamethrin (World Health Organization, 2007). Most research has evaluated the toxicity of LLINs but there been less publication on evaluating behavioral actions against mosquito vectors. A high-throughput screening system (HITSS) was developed by Grieco et al. (2005) as a novel assay system for rapid mass screening of chemical compounds. The HITSS has an integrated design that allows for examination of two behavioral responses (contact irritancy and spatial repellency) as well as toxicity response. A contact irritant action stimulates directed movement away from the chemical source after the mosquito makes physical contact. A spatial repellent action stimulates directed movement away from the chemical source after the mosquito detects a chemical gradient in the air. A toxic action (knockdown or death) results after the mosquito has contacted or non-contacted exposure with the chemical (Grieco et al., 2007). The aim of the current study was to evaluate the three chemical actions (contact irritancy, spatial repellency, toxicity response) of two types of alpha-cypermethrin LLINs—LN A (candidate net), a novel LLIN which is not recommended by the WHO and LN B (reference net), a LLIN which is recommended by the WHO—with unwashed and washed conditions against *Ae. aegypti* using the HITSS.

Materials and Methods

Mosquitoes

The *Ae. aegypti* sample used in this study was originally collected from Orange Walk, Belize, Central America. The mosquito colony was established from larvae collected in June 2015 and was susceptible to pyrethroid (Wagman et al., 2015). Species identification and colonization were carried out at the Faculty of Biological Science, Notre Dame University, South Bend, IN, USA. The *Ae. aegypti* colony was maintained following the standard protocol of Achee (2009). Paper containing eggs of *Ae. aegypti* was placed in hatching cups with 450 mL of tap water and placed under a vacuum for 15–30 min. After the eggs had hatched, fish pellets were placed in the larvae cups. The second stage larvae were sorted in groups (50 larvae per group) and were reared to the pupal stage under controlled conditions in incubators (28 °C, 80 ± 5% relative humidity (RH) and a 12 hr:12 hr light:darkness photoperiod). Pupae were then picked from rearing cups everyday using a standard plastic pipette. A size exclusion separator was used to separate the smaller-sized male pupae from the bigger females. The male pupae were reared for continue the colony, whereas 250 female pupae were used to conduct the experiment. Cotton pads soaked in a 10% sugar solution were provided to mosquitoes. Sugar-starved, nulliparous *Ae. aegypti* females aged 3–5 d were sorted into groups of 10 for contact irritancy assay (CIA) and of 20 each for the spatial repellency assay (SRA) and toxicity response assay (TOX), as previously described (Grieco et al., 2005). Water-soaked cotton pads were provided until 24 hr before testing.

Treatments

Two types of LLINs impregnated with alpha- cypermethrin were used for this study—LN A (candidate net) and LN B (reference net). Both LLINs were separated into two groups, unwashed and washed 20 times. Washing occurred at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, according to a protocol adapted from the standard WHO washing procedure used in Phase I (World Health Organization, 2013). Nets were washed in bowls containing 10 L of clean water with a maximum hardness of 5 dH and 2 g/L of soap ("savon de Marseille"-like) using manual agitation. Each net was agitated for 3 min, left to soak for 4 min and re-agitated for 3 min before rinsing twice with 10 L of clean water each time. Nets were dried horizontally in a shaded area and then stored at ambient temperature between washing periods, with washing once a day, and 20 washes for each LLIN. Each LLIN was cut in five pieces of 11 cm × 25 cm according to WHO guidelines (World Health Organization, 2013), as shown in Fig. 1 and was stored in dark place. The cut pieces of LLINs were used in the HITSS assay (Grieco et al., 2005). Untreated polyethylene nets were used for the control group.

High-throughput screening system

All assays were conducted during daytime in a fume hood at 24 ± 2 ºC and 47 ± 2% RH. The HITSS chambers were washed at the end of each testing day. The parts of chambers which were contaminated with the treated nets were washed using acetone while all other parts were washed using a detergent solution (Liqui-Nox; Aloconox; New York, NY, USA). Component sections were allowed to dry overnight before reuse.

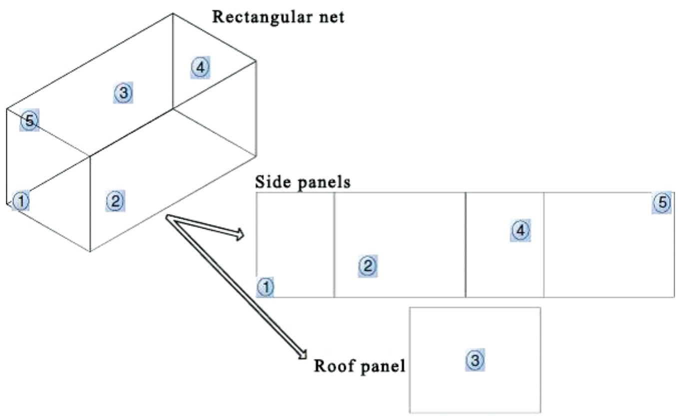

Fig. 1 Sample testing for compliance with specified recommended positions
Source: World Health Organization (2013)

Contact irritancy assay

The CIA device consisted of a clear cylinder and a metal chamber with the net inside (Fig. 2A). A butterfly valve between these chambers was used to control the flight movement of the tested mosquitoes. Before testing, 10 female mosquitoes were introduced into each plastic tube using a vacuum aspirator. After setting up the CIA, the 10 mosquitoes were transferred into the metal chamber and allowed to rest for 30 sec. Then, the butterfly valve was opened for 10 min. The clear cylinder was covered by a black cloth sheet while the valve was open. After 10 min, the butterfly valve was closed. The number of mosquitoes that flew into the clear cylinder (the number escaping) and the numbers of knockdown mosquitoes in the clear cylinder and the metal chamber were recorded (Grieco et al., 2007; Thanispong et al., 2010). Six replicates were performed for each LLIN and its control (Grieco et al., 2007; Thanispong et al., 2010).

Spatial repellency assay

The SRA device consisted of three chambers, with two being metal cylinders and the third a clear central cylinder (Fig. 2B). One metal cylinder contained the LLIN and the other metal cylinder contained untreated polyethylene nets. Butterfly valves connected both sides of the clear cylinder and the metal chambers. A sample of 20 mosquitoes was transferred into the clear central cylinder using air blowers and mosquitoes were allowed to rest inside for 30 sec while the butterfly valves were closed. After this period, both sides of the butterfly valves were opened for 10 min. The clear central cylinders were covered with black cloth sheets while the valves were open. After 10 min, the butterfly valves were closed. The numbers of mosquitoes within each chamber, as well as the number exhibiting a knockdown response, were recorded. Nine replicates were carried out with each treatment and control. Each system was rotated after the fourth replicate to minimize possible effects from positional bias (Grieco et al., 2007; Thanispong et al., 2010).

Toxicity response assay

TOX consisted of metal chambers only (control and treatment) fixed with an end cap and funnel section (Fig. 2C). Each metal chamber contained LLINs for the treatment group and untreated polyethylene nets for the control group. A sample of 20 mosquitoes was transferred into the metal chamber using air blowers and were exposed to the net for 60 min. After 60 min, the number of knockdown mosquitoes were recorded, and they were then transferred to holding cups and provided with 10% sugar solution pads. The holding cups were maintained at 25 ºC and 80 ± 5% RH for 24 hr. After 24 hr the mortality numbers of mosquitoes were recorded. Six replicates were performed for each LLIN and its control (Grieco et al., 2007; Thanispong et al., 2010).

Data analysis

Contact irritancy assay data were analyzed using the Wilcoxon two-sample test (SAS Institute, 1999) to determine the difference between the numbers escaping from treated and control chambers. Percentage escaping per replication were analyzed for each LN by two-way analysis of variance (ANOVA) with LLIN and position of LLIN. Mean percentage escaping was dependent variable corrected for the number escaping in the control and KD in the metal chambers.

Spatial repellency assay data were analyzed using a nonparametric signed-rank test (SAS Institute, 1999) to determine whether the mean spatial activity index (SAI) for each treatment was significantly different from zero. The SAI evaluated repellent responses of mosquitoes which calculated by SAI = $(N_c - N_t)/(N_c + N_t)$, where N_c is the numbers of mosquito in control chamber and N_t is the numbers of mosquito in treatment chamber. The SAI values range from -1 to 1, SAI value -1 indicates an attractant response (mosquito moved to treatment chamber), SAI value 1 indicates a repellent response (mosquito moved to control chamber) and SAI value 0 indicates no attractant or repellent response. For the toxicity response data, percentage knockdown and mortality values were corrected using Abbott's formula (Abbott, 1925) and transformed to arcsine square root values for analysis of variance (Grieco et al., 2005; Thanispong et al., 2010).

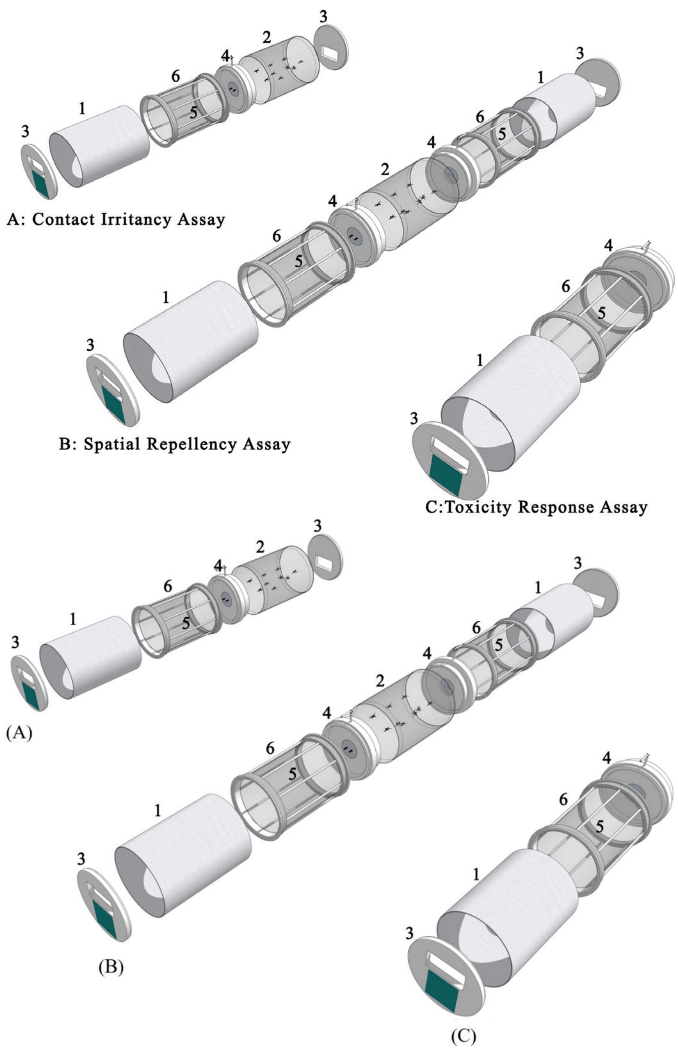

Fig. 2 Schematic drawing of high-throughput screening system showing components parts of: (A) contact irritancy assay; (B) spatial repellency assay; (C) toxicity response assay, where 1 = treatment (metal) cylinder, 2 = clear (Plexiglas) cylinder, 3 = end cap, 4 = linking section, 5 = treatment drum, 6 = treatment net

Results

The contact irritant, spatial repellent and toxicity responses of *Ae. aegypti* were observed when tested against either washed or unwashed alpha-cypermethrin LLIN material. There was a significantly ($p = 0.0022$) high contact irritant response of *Ae. aegypti* when exposed to all LLINs. In contrast, no spatial repellent response was observed for any of the LLIN test materials.

Contact irritant responses

High irritancy of mosquitoes was observed against all five different positions of the four LLIN tested materials (unwashed LN A, washed LN A, unwashed LN B, washed LN B). A high mean number of escape mosquitoes was obtained, ranging between 2.33 ± 0.90 and 5.83 ± 1.32. The highest response of escape mosquitoes was from LN B washed at the third position (the middle of the roof panel of the LLIN) (5.83 ± 1.32) which showed the mean highest percentage of escape is 94.17 % whereas the LN B washed at the first position showed higher number escaping (6.66 ± 0.30) than LN B washed at the third position but LN B washed at the third position showed mean highest percentage of escape. The lowest response was observed from the LN A unwashed at the first position (2.33 ± 0.90) (the bottom left corner of the wide side of the LLIN) which showed the lowest mean percentage of escape is 49.25% whereas the LN A washed at the second position showed lower number escaping (2.16 ± 0.45) than LN A unwashed at first position but LN A unwashed at first position showed lowest mean percentage of escape. A significant ($p = 0.0022$) difference in number of mosquito escapees was recorded between overall treatments (LNs) and untreated controls (untreated polyester nets), as shown in Table 1.

Spatial repellent responses

No spatial repellent response of *Ae. aegypti* mosquitoes was observed when tested against all positions of the four LLIN materials (Table 2). With this assay, washed LN A at the first position (the bottom left corner of the wide side of the LLIN) had the highest mean number of mosquitoes escaped to control chamber (2.78 ± 1.72). The mean spatial activity index of all LLINs showed very low repellent response in range -0.04 ± 0.49 to 0.83 ± 0.24.

Toxicity responses

Toxicity action data from all alpha-cypermethrin LLIN materials against *Ae. aegypti* mosquitoes were comparatively high, ranging between 57.66% and 100%. The highest mean percentage knockdown and mortality were observed from washed LN B (the reference net). Washed LN B at the first position (the bottom left corner of the wide side of the LLIN) had the highest percent mortality (100%). The lowest mean percent knockdown and mortality were observed from unwashed LN A (the candidate net). Unwashed LN A at the third position (the middle of the roof panel) had the lowest percentage mortality (57.66 ± 3.10%), as shown in Table 3.

Discussion

The chemicals used in vector intervention programs place more attention on their killing ability than to nontoxic action due mainly to the unavailability of an acceptable and practicable testing system to identify the entire series of chemicals actions (Roberts et al., 1997; Grieco et al., 2007; Chareonviriyaphap et al., 2013). Such a complete series includes insecticidal, irritant and repellent. The first action is used for compounds that can kill the insect, whereas the last two are known as 'excito-repellency' that chase mosquitoes away from the treated area. Irritancy occurs when mosquitoes leave the treated area after making physical contact with the chemical. In contrast, repellency takes place when mosquitoes depart the treated area prior to contact with the insecticide. The excito-repellency test system is considered the gold standard for studying mosquito behavior to chemicals.

Table 1 Mean (± SD) responses of female *Aedes aegypti* in contact irritancy assay to long-lasting insecticidal nets (LLINs) by type, wash condition and different positions

LLIN type	Position	Number escaping		*p* value
		Treatment	Control (untreated polyester net)	
LN A unwashed	1	2.33±0.90	0.33±0.31	0.0022
	2	2.83±1.26	0.66±0.24	0.0022
	3	4.5±1.62	0.66±0.45	0.0022
	4	4.16±0.87	0.33±0.31	0.0022
	5	4.67±1.27	0.33±0.31	0.0022
LN A washed	1	6.5±0.82	0.33±0.48	0.0022
	2	2.16±0.45	0.16±0.61	0.0022
	3	4±0.75	1.16±0.48	0.0022
	4	5.5±1.17	0.33±0.31	0.0022
	5	5.33±0.89	0.33±0.31	0.0022
LN B unwashed	1	4.17±0.79	0	0.0022
	2	5.33±1.38	0	0.0022
	3	4.67±0.89	0.5±0.50	0.0022
	4	4±1.40	0.5±0.50	0.0022
	5	3.83±1.21	0.5±0.32	0.0022
LN B washed	1	6.66±0.30	0.16±0.24	0.0022
	2	4.17±1.37	1.33±0.61	0.0022
	3	5.83±1.32	0	0.0022
	4	4.16±1.09	0.5±0.50	0.0022
	5	5.33±0.97	0.5±0.32	0.0022

p-values are from Wilcoxon two-sample test for difference between the number escaping in treated LLINs and untreated polyester net (control).

Table 2 Number of mosquitoes escaping (mean ± SD) in control chamber and treatment chamber and mean spatial activity index (SAI, mean ± SD) of female *Aedes aegypti* in the spatial repellency assay to different long-lasting insecticidal nets (LLINs)

LLIN type	Position	number of mosquitoes in control chamber	number of mosquitoes in treatment chamber	*p*-values	Mean SAI
LN A unwashed	1	1.22±0.83	0.56±0.72	0.25	0.389±0.56
	2	0.78±0.83	0.33±1	0.55	0.4±0.40
	3	1.11±0.6	1.22±1.85	0.71	-0.04±0.49
	4	0.22±0.44	0.33±0.71	0.71	0.33±0.34
	5	0.44±0.72	0±0	0.12	0.41±0.33
LN A washed	1	2.78±1.72	0.11±0.33	0.11	0.83±0.24
	2	0.89±1.45	0.67±1.12	0.58	0.01±0.47
	3	0.78±1.09	0.11±0.33	0.13	0.33±0.47
	4	1.44±1.74	0.56±1.01	0.11	0.24±0.45
	5	0.78±0.66	0.89±1.36	0.91	0.12±0.49
LN B unwashed	1	1.56±1.13	0±0	0.17	0.77±0.29
	2	0.67±0.86	0.22±0.67	0.19	0.30±0.36
	3	1±1.5	1.22±1.64	0.86	-0.07±0.60
	4	0.78±1.09	0.78±0.97	1	0 ±0.47
	5	0.78±0.67	0.56±0.72	0.48	0.22±0.57
LN B washed	1	1.11±1.67	0.33±0.71	0.1	0.33±0.47
	2	0±0	0.11±0.33	0.32	-0.11±0.22
	3	0.56±0.73	1±1.22	0.51	-0.07±0.53
	4	1.56±1.33	0.3±0.7	0.04	0.57±0.36
	5	1.56±-.73	0.67±0.86	0.46	0.48±0.35

p-values are from Wilcoxon two-sample test for difference between the number escaping in treated LLINs and untreated polyester net (control).

Table 3 Percentage knockdown and mortality (mean ± SD) of female *Aedes aegypti* in toxicity assay for different long-lasting insecticidal nets (LLINs)

LLIN type	Position	% Knockdown	% Mortality
LN A unwashed	1	39.72±3.33	78.65±2.07
	2	52.73±5.07	61.21±6.40
	3	42.52±4.02	57.66±3.10
	4	64.86±9.32	75.75±6.11
	5	56.75±4.33	66.14±9.31
LN A washed	1	77.38±7.74	77.38±7.65
	2	74.39±9.39	74.39±9.39
	3	85.69±3.42	86.52±4.03
	4	85.10±2.17	82.45±3.97
	5	84.00±5.06	80.92±5.94
LN B unwashed	1	87.81±2.94	90.27±2.36
	2	91.43±4.01	91.38±3.63
	3	84.09±3.54	86.49±5.00
	4	96.57±1.58	96.61±1.55
	5	87.80±3.74	77.46±13.00
LN B washed	1	97.41±1.68	100
	2	89.56±4.82	99.17±1.21
	3	89.32±4.04	87.71±4.60
	4	91.03±4.01	96.63±1.57
	5	99.20±1.15	99.01±1.42

The long lasting insecticide treated nets (LLINs), with synthetic pyrethroids incorporated into the net fabric results in longer lasting action of the chemical insecticide (World Health Organization, 2007); these have been used as the standard method for malaria prevention in Africa south of the Sahara and in many Asian countries, as well as in dengue-endemic areas (Malima et al., 2013). The LLINs have been evaluated on toxicity to vectors, serviceable life duration under operational conditions. Several studies (Lengeler, 2004; Ntonifor and Veyufambom, 2016) have reported on the use of LLINs to protect humans from mosquito bites. In those studies, the LLINs were prepared in the form of door and window curtains, mosquito nets, or other physical barriers. However, none of these studies investigated the chemical actions of the LLIN products.

The current study evaluated the impact of two different types of LLINs—LN A (candidate net) and LN B (reference net) — on *Ae. aegypti* using the high-throughput screening system (HITSS) to determine the two behavioral responses (contact irritancy and spatial repellency) as well as toxicity response. A higher number of escaping (contact irritancy) *Ae. aegypti* was observed following exposure to LN B than LN A, with no spatial repellency. These results indicated that the reference net recommended by the WHO had greater efficacy than the candidate net. However, both LLINs produced high mortality on the mosquito population, which agreed with the studies by Grieco et al. (2007) and Thanispong et al. (2009). Furthermore, the primary action of alpha-cypermethrin, which was coated on the treated nets, is via contact irritancy, while toxicity is its secondary action (Achee et al., 2009).

The results of the toxic response in the current study indicated that all LLINs produced a high percentage mortality. However, LN B was more effective regarding toxicity than LN A. In addition, the knockdown effect of LN B was higher than LN A. The results of SRA showed only one position that have repellent action in very low percentage indicating that the aim of LN was success because when mosquitoes contacted to the LN then they flew away and died. The WHOPES recommended at least 20 standard washes of LLINs to extend their biological activity (World Health Organization, 2007). The novel LLINs in the current study produced similar biological activity in both the unwashed and washed groups.

LLINs have been promoted by the WHO as a primary strategy in the control of malaria vectors in malaria-endemic areas. However, some malaria-endemic areas are also dengue-endemic. Nowadays people have multiple occupations, with some during the night or early morning, for example rubber workers maintaining and harvesting latex from plantations, who work during the early morning and sleep during the day time with a high probability of being exposed to mosquitoes feeding (Reiter et al., 1991). Such people are at risk of contracting both malaria and dengue. Therefore, the broader application of LLINs could protect these workers from both mosquito vectors. This study demonstrated the importance of identifying all modes of action for the proper selection of vector control tools, such as the use of LLINs against mosquito-borne diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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